Note

Effect of varying protein levels on growth and digestive enzyme activities of pengba *Osteobrama belangeri* (Valenciennes, 1844)

R. RAMESH¹, K. DUBE², A. K. REDDY², P. V. RANGACHARYULU¹,
G. VENKATESHWARLU² AND P. JAYASANKAR³

¹Regional Research Centre of ICAR-Central Institute of Freshwater Aquaculture, Fish Seed Farm, Penamalururu Poranki P. O., Vijayawada - 521 137, Andhra Pradesh, India

²ICAR-Central Institute of Fisheries Education, Panch Marg, Off Yari Road, Versova, Andheri (West) Mumbai - 400 061, Maharashtra, India

³ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar - 751 002, Odisha, India
e-mail: rathodcifa@gmail.com

ABSTRACT

Pengba, *Osteobrama belangeri* (Valenciennes, 1844) is considered as one of the candidate species for diversification of aquaculture. However, only scanty information is available on its nutrient requirements. Hence, the present study was conducted on growth and digestive enzyme activities of pengba fry in response to different dietary protein levels. Six semi-purified isocaloric diets were formulated with different levels of protein at 5% increments (25, 30, 35, 40, 45 and 50%) and fed to different groups of pengba fry for a period of 60 days. Survival of fish was significantly influenced by different protein levels ($p < 0.05$). The highest weight gain% and specific growth rate ($4.17\% \text{ day}^{-1}$) was obtained at 45% protein level which thereafter declined. The best feed conversion ratio (1.46) was observed at 45% protein level and the highest protein efficiency ratio (2.14) was obtained at 25% protein level with significant difference ($p < 0.05$). The crude protein content of fish was significantly affected by dietary protein levels ($p < 0.05$) and highest was recorded at 45% protein diet. Moisture, ether extract and ash content did not show significant ($p > 0.05$) variation among dietary treatments. The activity of proteolytic enzymes (proteases, trypsin and chymotrypsin) and acid phosphatase were significantly ($p < 0.05$) enhanced by dietary protein levels up to 45%, which were found to decline thereafter. Amylase activity was significantly decreased as protein levels increased in the diet. Lipase and alkaline phosphatase activities were not influenced by dietary protein levels. In the present study, pengba fry fed with 45% protein diet, showed the best growth performance and highest digestive enzyme activities. The activity of digestive enzymes in the gut clearly demonstrated that the proteolytic enzymes are activated in response to dietary protein utilisation and also revealed that pengba fry may require high protein (45%) diet for better growth and survival. Second order polynomial regression analysis revealed that the maximum protein requirement of pengba fry was 45.1% ($r^2 = 0.90$). The findings of the study is expected to have significant implications for feed development for pengba aquaculture.

Keywords: Digestive enzymes, Growth, *Osteobrama belangeri*, Pengba, Protein levels

Protein is very much essential for growth and to maintain the metabolic activities of fish (Wilson and Halver, 1986). The essential amino acids (EAA) and non-essential amino acids (NEAA) incorporated in the fish feed help to synthesise body protein and provide energy for metabolic activity (Lovell, 1989; Kaushik *et al.*, 1995). Studies on protein requirements of carps have been undertaken by many workers using casein based synthetic diets (Mahanta *et al.*, 2008; Ismat *et al.*, 2013). Adequate amino acid levels required to maximise the growth of fish is supplied through optimum dietary protein levels (Siddiqui and Khan, 2009). Excess protein in the diet increases the production cost and can lead to pollution of pond environment. Therefore, it is necessary to optimise the protein levels in the fish diets considering

economics and environmental perspective (Siddiqui and Khan, 2009; Akpınar *et al.*, 2012).

The activities of digestive enzymes may vary among different fish species, which mainly depends on age of fish, quantity and composition of the diet (Peres *et al.*, 1998). Digestive enzyme activities of different fish species have been reported with varying levels of dietary proteins (Debnath *et al.*, 2007; Mahanta *et al.*, 2008; Bhilave *et al.*, 2014). Activities of digestive enzymes and nutrient utilisation are the key factors that help in standardisation of fish feeding protocols (Doos Ali Vand *et al.*, 2014). Patterns of digestive enzyme activity can reflect the feeding habits as well as digestive capacity of fish and these activities are modulated based on the source, quality and quantity of the feed (Debnath

et al., 2007; Gonza'lez-Fe'lix *et al.*, 2010). Further, it helps in predicting the possibilities of utilising different nutrients in the diet of fish (Hofer and Kock, 1989).

Pengba, *Osteobrama belangeri* (Valenciennes, 1844), is a minor carp which belongs to cyprinid group and it is distributed in the rivers and lakes of Asian countries like Yunnan Province of China, Myanmar and India. Pengba is regarded as the state fish of Manipur. The fish has been identified as a potential candidate species for diversification of aquaculture in India as it is a preferred species due to its high quality meat. However, low survival of juveniles is one of the major limitations for its adoption. Earlier, information on food and feeding habits and growth in relation to dietary protein utilisation of pengba was reported by Basudha *et al.* (1999, 2001). However, there is no information available on effect of different protein levels on the digestive pattern of this species. The survival and growth of fish mainly depend on nutritionally balanced feed and knowledge on digestive physiology is essential to understand nutritional strategies for the formulation of such fish diets. With this background, the present study was carried out in order to study the effect of varying protein levels on growth performance and to assess the digestive enzymes activity of pengba fry in response to protein utilisation.

Six isocaloric experimental diets were prepared with different levels of protein *viz.* 25, 30, 35, 40, 45 and 50%. List of ingredients and proximate composition of the diets is presented in Table 1. Semi-purified diets were used in the

study with casein and gelatin as protein source (maintained at a ratio of 4:1) and sunflower oil and fish oil were used as lipid source in equal proportions. Energy was adjusted with dextrin and starch. α -Cellulose was used as filler component.

Three hundred sixty nos. (20 per tank) of pengba fry (mean weight of 0.45 ± 0.01 g) were stocked in 18 tanks of 100 l capacity. Each diet was randomly assigned to triplicate tanks. Fish were fed initially at 6% of body weight and later reduced to 3% of body weight. Experimental feeding was done for a period of 60 days which was given daily at 09 00 and 17 00 hrs. All the tanks were siphoned to remove waste materials to maintain healthy environment in the tank and $\frac{1}{4}$ th water exchange was done every day. All the water quality parameters were recorded at every fortnight. The fish were starved for 24 h before sampling.

Experimental diets and fish carcass were analysed as per AOAC (2003) for proximate composition. Moisture content was analysed by oven drying at 105°C for 24 h; crude protein content was determined by Kjeldahl method (Nx6.25); ether extract was estimated with diethyl ether for 6 h in a soxhlet apparatus. Muffle furnace was used to estimate the ash content from moisture free samples at 600°C for 12 h. The nitrogen free extract (NFE) was estimated by subtracting the contents of protein, fat, ash and moisture.

Growth and FCR was determined using the following formulae:

Survival (%) = $100 \times \text{Final no. of fish (FN)} / \text{Initial no. of fish (IN)}$

Weight gain (%) = $[(\text{Weight of fish at final day (FW)} - \text{Initial weight of fish (IW)}) / \text{Initial weight}] \times 100$

Table 1. Ingredients used and nutrient composition of the experimental feeds (% dry matter)

Ingredient composition	Protein levels (%)					
	25	30	35	40	45	50
Casein	23.9	28.68	33.46	38.85	43.39	48.66
Gelatin	6	7.2	8.4	9.1	10.5	11.3
Starch	16.65	14.85	13.5	12.5	11.23	9.45
α -cellulose	8.35	9.15	9.5	10.5	10.77	11.55
Dextrin	35.1	30.12	25.14	19.05	14.11	9.04
CMC ¹	2	2	2	2	2	2
Fish oil	3	3	3	3	3	3
Sunflower oil	3	3	3	3	3	3
Vitamin-mineral mix ²	2	2	2	2	2	2
Proximate composition						
Dry matter	94.76	94.37	93.71	95.27	96.02	96.11
Crude protein	25.02	30.49	34.98	40.66	44.94	50.01
Ether extract	6.14	6.30	6.53	5.95	6.18	6.16
Ash content	5.18	5.67	6.01	5.04	5.9	5.1
Nitrogen free extract	48.42	41.91	37.19	34.52	32.3	26.23

¹Carboxy methyl cellulose

²Vitamin-mineral mix (Debnath *et al.*, 2007)

Specific growth rate (SGR, % day⁻¹) = [ln FW - ln IW] / No. of culture days x 100

Food conversion ratio (FCR) = Dry feed intake (g) / Wet weight gain (g)

Feed efficiency ratio (FER) = Net wet weight gain (g) / Dry feed intake (g)

Protein efficiency ratio (PER) = (Final weight - Initial weight) / (Total feed consumed by fish x Protein level in the diet).

In each treatment, six fishes were used for the enzyme analysis and their intestine were dissected and frozen immediately at -20°C for enzyme assays following standard protocol (Debnath *et al.*, 2007).

The activity of proteolytic enzyme (proteases, trypsin and chymotrypsin) was estimated by the casein digestion method of Kunitz (1947). Lipase activity was determined based on Cherry and Crandell (1932), whereas, starch solution was used as substrate for the analysis of amylase (Rick and Stegbauer, 1974). In addition, Garen and Levinthal (1960) method was used to determine the activities of alkaline phosphatase (ALP) and acid phosphatase (ACP).

One-way analysis of variance (ANOVA) was used for data analysis. Duncan's multiple range test (DMRT) was performed to measure significant difference between the treatments (SPSS, Version 16.0).

The observations of final weight, survival, weight gain% and SGR of fish fed with different treatments are given in the Table 2. Average weight gain percentage was significantly ($p < 0.05$) higher in fish fed with 45% of protein level and lowest was found at 25% dietary protein. The highest SGR ($4.17 \pm 0.01\%$ day⁻¹) was noticed with 45% of protein diet and lowest ($3.34 \pm 0.01\%$ day⁻¹) was observed with 25% protein diet. The final weights, weight gain% and SGR increased significantly ($p < 0.05$) with increase in dietary protein levels upto 45% and thereafter decreased. The survival rate varied from 72.45 to 89.42% and significantly affected ($p < 0.05$) by dietary protein levels. The second order polynomial regression analysis ($y = -0.002x^2 + 0.179x + 0.06$;

$R^2 = 0.90$) showed that the optimum protein levels to achieve high growth rate of pengba fry was 45.1% (Fig. 1).

Significant difference ($p < 0.05$) was observed between the treatments in response to the feed utilisation parameters (FCR and PER) (Table 2). The best FCR value (1.46 ± 0.01) was found in fish fed 45% protein level followed by 40% protein with no significant difference ($p > 0.05$) between these two treatments. The highest feed efficiency was observed with 45% dietary protein which was similar ($p > 0.05$) to the 40% protein level, but was significantly ($p < 0.05$) higher compared to other dietary treatments. As dietary protein level increased, there was significant decrease ($p < 0.05$) in the PER value.

Carcass composition of fish was influenced by the different protein levels (Table 3). Crude protein, moisture, ether extract and ash content at different dietary protein levels were significantly different ($p < 0.05$). The moisture content of fish was not significantly ($p > 0.05$) different among the dietary treatments. Crude protein content ($14.95 \pm 0.01\%$) was found highest in fish fed with 45% of dietary protein level and lowest ($12.83 \pm 0.02\%$) was observed in 25% protein level.

Fig. 1. Estimation of dietary protein requirement of *Osteobrama belangeri* fry by second order polynomial regression analysis

Table 2. Growth, survival rate and feed parameters of *Osteobrama belangeri* fed at different protein levels

Parameters	Protein levels (%)					
	25	30	35	40	45	50
Initial weight (g)	0.45± 0.01	0.45±0.01	0.45±0.01	0.45±0.01	0.45±0.01	0.45±0.01
Final weight (g)	3.34±0.02 ^a	3.85±0.04 ^b	4.27±0.08 ^c	5.18±0.04 ^c	5.51±0.04 ^f	4.69±0.03 ^d
Weight gain (%)	643.70±6.58 ^a	755.56±10.18 ^b	850.37±19.64 ^c	1052.60±9.72 ^c	1124.40±8.98 ^f	943.70±7.06 ^d
SGR (% day ⁻¹)	3.34±0.01 ^a	3.57±0.01 ^b	3.75±0.03 ^c	4.07±0.01 ^c	4.17±0.01 ^f	3.90±0.01 ^d
FCR	1.86±0.02 ^c	1.73±0.01 ^b	1.72±0.04 ^b	1.50±0.01 ^a	1.46±0.01 ^a	1.68±0.02 ^b
FER	0.53±0.01 ^a	0.57±0.01 ^b	0.58±0.01 ^b	0.66±0.01 ^c	0.68±0.01 ^c	0.59±0.01 ^b
PER	2.14±0.07 ^c	1.92±0.04 ^d	1.66±0.03 ^c	1.65±0.02 ^c	1.52±0.02 ^b	1.18±0.02 ^a
Survival rate (%)	72.45±0.05 ^a	78.36±0.31 ^b	79.47±0.22 ^c	86.23±0.14 ^c	89.42±0.09 ^f	82.75±0.23 ^d

Results are presented as mean± SE (n=3). Mean values with different superscripts in the same row are significantly different ($p < 0.05$)

Table 3. Proximate composition of *Osteobrama belangeri* fed at different dietary protein levels (% wet weight basis)

Nutrient composition	Protein levels (%)						
	Initial	25	30	35	40	45	50
Moisture	79.6±0.06	79.02±0.01 ^a	78.89±0.01 ^a	78.65±0.02 ^a	78.32±0.04 ^a	78.61±0.14 ^a	78.33±0.05 ^a
Crude protein	12.76±0.02	12.83±0.02 ^a	13.50±0.03 ^b	13.94±0.02 ^d	14.12±0.01 ^c	14.95±0.01 ^f	13.62±0.01 ^c
Ether extract	1.94±0.03	2.57±0.01 ^a	2.42±0.01 ^a	2.34±0.02 ^a	2.25±0.02 ^a	2.04±0.01 ^a	2.17±0.01 ^a
Ash	3.01±0.02	3.72±0.01 ^a	3.53±0.01 ^a	3.61±0.01 ^a	3.67±0.02 ^a	3.29±0.03 ^a	3.92±0.02 ^a

Results are presented as mean± SE (n = 3). Mean values with different superscripts in the same row are significantly different (p<0.05)

Moisture, ether extract and ash content were not influenced by dietary protein levels and they did not vary significantly among dietary treatments.

The proteolytic enzymes (proteases, trypsin and chymotrypsin) and ACP activities were significantly (p<0.05) different among dietary treatments (Table 4). Fish fed with 45% protein level was found to have the highest protease, trypsin and chymotrypsin activities and lowest activity was found in fish fed at 25% protein level. As dietary protein level in the diet was raised up to 45% the activities of proteolytic enzymes were found to increase and thereafter declined significantly (p<0.05) with further increase in protein levels.

Significant difference (p<0.05) in the activity of amylase was observed with different dietary treatments. Though the activities of lipase and ALP slightly increased with increase in dietary protein levels, the difference was not significant (p>0.05). An increasing trend was observed in the activity of ACP which responded significantly (p<0.05) to increase in dietary protein levels, except in 50% protein diet, where the activity of enzyme was decreased.

It is well known that protein is an expensive and the most important nutrient for feed formulation (Wee and Tacon, 1982; Zehra and Khan, 2011). In the present study, growth (weight gain and SGR) and survival of pengba increased significantly (p<0.05) with increase in dietary protein levels from 25 to 45%, and thereafter decreased at 50% protein level. Similar

results were observed in various fish species such as *Labeo rohita* (Debnath *et al.*, 2007); *Puntius gonionotus* (Mahanta *et al.*, 2008) where the growth levels varied with different levels of protein content in the diets.

In the present study, the best growth performance, survival and feed utilisation of fish were achieved at 45% protein level than in other dietary treatments. From the results of the present study, it was inferred that the feed with 45% protein level was utilised efficiently and resulted in maximum growth (p<0.05) and had lower FCR. These results are supported by earlier study that protein requirement of *Carassius auratus* was between 40-55% (Fiogbe and Kestemont, 1995). Further, the present findings are supported by Basudha *et al.* (2001) who reported that growth performance of pengba was higher in fish fed with 40% protein level. However, protein utilisation of pengba observed in the present study is higher than the range determined in the common carp *Cyprinus carpio* (35-42%) (Tacon, 1987).

Lesser protein content in the fish diets always results in negative impact on growth. Maximum protein in the diet is used for growth (protein synthesis) and the remaining protein which is unutilised, will be transformed as waste. In the present study, fish growth was found lowest in fish fed with 25 to 30% protein levels, as most of the protein in the diet was used for metabolic activity and repair

Table 4. Digestive enzyme activity in the intestine of *Osteobrama belangeri* fed at different protein levels

Enzymes	Protein levels (%)					
	25	30	35	40	45	50
Protease	8.38±0.36 ^a	9.71±0.34 ^b	12.11±0.23 ^c	16.41±0.35 ^c	20.56±0.28 ^f	14.18±0.09 ^d
Trypsin	6.57±0.06 ^a	8.33±0.01 ^b	10.49±0.09 ^d	12.35±0.03 ^c	14.48±0.12 ^f	9.85±0.02 ^c
Chymotrypsin	4.61±0.02 ^a	6.40±0.03 ^b	9.62±0.03 ^c	11.18±0.08 ^d	13.48±0.09 ^c	9.45±0.02 ^c
Amylase	14.18±0.01 ^c	12.91±0.01 ^{bc}	12.42±0.07 ^{bc}	11.99±0.39 ^b	11.66±0.30 ^b	9.60±0.30 ^a
Lipase	0.66±0.01 ^a	0.65±0.05 ^a	0.73±0.01 ^a	0.75±0.02 ^a	0.81±0.04 ^{ab}	0.73±0.03 ^a
ALP	12.34±0.08 ^a	12.59±0.05 ^a	13.06±0.02 ^a	13.42±0.05 ^a	13.49±0.30 ^a	13.19±0.02 ^a
ACP	8.77±0.04 ^a	10.65±0.21 ^b	13.37±0.07 ^c	17.70±0.18 ^c	20.37±0.09 ^f	16.93±0.01 ^d

Proteolytic enzymes expressed in micromol of tyrosine released per min per g protein ($\mu\text{m tyrosine min}^{-1} \text{ g protein}^{-1}$); amylase expressed as $\mu\text{m maltose min}^{-1} \text{ g protein}^{-1}$; lipase quantified as units mg protein⁻¹; ALP and ACP are expressed in nanomoles p-nitrophenol released ($\text{nm p-nitrophenol min}^{-1} \text{ mg protein}^{-1}$) at 37°C; Means with different superscripts in the same row are significantly different (p<0.05).

making it unavailable for growth. Although, the growth indices increased significantly ($p < 0.05$) with increase in dietary protein levels up to 45%, the same were found to decrease at 50% protein level. These findings were supported by Mohanta *et al.* (2008) who reported that growth rate may vary at higher protein levels in *Puntius gonionotus*. When a high protein diet is supplied, mostly it gives rise to energy for maintenance of the body (Winfree and Stickney, 1981), which ultimately reduces protein utilisation in fish (Lee and Putnam, 1973).

FCR and PER significantly ($p < 0.05$) improved from 25 to 45% protein levels. Several studies showed that whenever high levels of protein is supplied in the diet, the level of nutrient utilisation decreased, and feed consumption is reduced. The requirement of nutrients for optimum growth are always more than the requirement for optimal production (Siddiqui and Khan, 2009; Hossain *et al.*, 2012). FCR was found to be the lowest at 45% protein level compared to all other dietary treatments. It is assumed that the optimum utilisation of protein and maximum growth in pengba could be achieved at 45% protein level. Further, as protein levels increased above 45%, the FCR value increased. It might be due to poor utilisation of dietary protein at higher protein levels and imbalance of protein to energy ratio. These findings are similar to the reports of Salhi *et al.* (2004) in *Rhamdia quelen* and Mohanta *et al.* (2008) in *Puntius gonionotus*. PER was found to decrease significantly ($p < 0.05$) as the protein level in the diet increased. The present study supports the findings of other workers in rohu (Gangadhara *et al.*, 1997), catla (Murthy and Naik, 2000) and olive flounder (Kim *et al.*, 2004).

In the current study, significant variation in crude protein content of fish ($p < 0.05$) was observed in fish fed different dietary protein levels, which revealed that conversion and deposition of protein in the whole body increased with an increase in dietary protein level. The results of the study is supported by findings of several researchers in different fish species (Lazo *et al.*, 1998; Doos Ali Vand *et al.*, 2014). Insignificant different variation in moisture, ether extract and ash content of fish between the dietary treatments was recorded. The results were supported by earlier studies on pengba fingerlings (Basudha *et al.*, 2001, 2002). In earlier studies, ether extract and moisture content showed inverse relationship in rohu fed with varying dietary protein levels (Debnath *et al.*, 2007). In the present study, protein levels of fish did not show any effect on ether extract and moisture contents and no such correlation was observed.

Growth pattern and protein levels in the diet are negatively correlated (Kohla *et al.*, 1992), which depends on various biological and environmental factors. Therefore, to study the optimum nutrient requirement of fish, profile of digestive enzyme activities are often studied (Debnath *et al.*, 2007; Gonza'lez-Fe'lix *et al.*, 2010). Therefore, in the present study, digestive capacity of pengba was studied to optimise the dietary protein levels. Significant ($p < 0.05$) changes were noticed in the digestive enzyme activities in the intestine of pengba in relation to dietary protein levels, with some exceptions.

The proteolytic enzyme activity of juveniles of pengba was found to increase at dietary protein levels above 25%. Significant ($p < 0.05$) increase in the activity of protease, trypsin and chymotrypsin was recorded with increase in dietary protein levels. In contrast, Lopez-Lopez *et al.* (2005) reported that protease activity and dietary protein levels were not correlated with growth performance of fish. In seabass juveniles, the activity of trypsin was found to be unresponsive to protein diets (Cahu and Zambonino Infante, 1994). The activities of proteolytic enzymes increased with increase in dietary protein up to 45% and thereafter declined in fish fed with 50% protein diet. The results are in line with the earlier reports that high acid proteolytic activity of gut content of sea bream (*Diplodus puntazzo*) juveniles can be correlated to the high protein content (Savona *et al.*, 2011) and the presence of highly indigestible carbohydrate content in the diet which further reduced the proteolytic activity (Falge *et al.*, 1978, Mohanta *et al.*, 2008).

In general, protein hydrolysis is effected by proteases subsequently combined with trypsin and chymotrypsin when the food reaches intestine. Trypsin and chymotrypsin are activated by trypsinogen and chymotrypsinogen in the intestine which are involved in the digestion of food particles (Ueberschar, 1993). In the present study increase in trypsin and chymotrypsin activity with respect to increase in protein level in the diet, demonstrated that these two enzymes are highly specific and helped in digestion of protein content in the intestine of pengba.

In general, amylase activity will be highest in and lower in carnivorous fishes (Sabapathy and Teo, 1993; Hidalgo *et al.*, 1999). The activity of amylase was significantly high ($p < 0.05$) at 25% level of dietary protein and it decreased with increase in protein levels. The results were in contrast to earlier studies of Plantikow (1981) in rainbow trout (*Oncorhynchus mykiss*) and Debnath *et al.* (2007) in rohu (*Labeo rohita*) who reported that amylase activity was found to increase in fish fed with increased levels of dietary protein. The decreased

levels of carbohydrates with increase in dietary protein levels, could be attributed to the decreased activity of amylase in the present study. The results are supported by the findings of Mohanta *et al.* (2008) in *P. gonionotus* fingerlings and Das and Tripathi (1991) in grass carp, *Ctenopharyngodon idella*. When the optimal level of protein in the diet is exceeded, enzyme activities slowly decrease (Ceccaldi, 1997). The activity of amylase is always influenced by other factors such as gut content (Kohla *et al.*, 1992; Bhilave *et al.*, 2014), the feeding habit (NRC, 1993; Sabapathy and Teo, 1993; Ismat *et al.*, 2013), temperature and season (Kuz'mina *et al.*, 1996) than the carbohydrate sources.

Lipase activity varied with different dietary protein levels. The activity was found to be within the range of 1.22-1.52 units mg protein⁻¹ and was insignificantly ($p>0.05$) different among dietary protein levels. Similar results were found in the studies conducted by Lundstedt *et al.* (2004) in juvenile pintado and Debnath *et al.* (2007) in rohu. Moreover, lipase activity was found higher in carnivorous fish as they consume rich fat in their diet (Chakrabarti *et al.*, 1995).

Activity of alkaline phosphatase (ALP) is an indicator for nutrient absorption in epithelial layers (Harpaz and Uni, 1999; Gawlicka *et al.*, 2000). Pengba has lesser ALP activity when compared to other herbivorous fishes (Harpaz and Uni, 1999). In this experiment, ALP activity was not significantly influenced by the different dietary treatments, but slightly increased with increase in dietary protein levels and similar results have been reported in Atlantic cod (Lemieux *et al.*, 1999). The activity of acid phosphatase (ACP) showed significant variations and increased gradually with increase in dietary protein levels. The increased growth rate and ACP activity of fish positively responded with increase in dietary protein levels and similar results of increasing activity of ACP in rohu fed high protein diets have been reported by Debnath *et al.* (2007).

The enhanced proteolytic enzyme activities in response to protein utilisation observed in the present study, clearly demonstrated that early fry of pengba required high protein diet for optimum growth and survival.

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